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Investigation of the Synergetic Antimicrobial Activity of PLGA-Methicillin on Methicillin Resistance *Staphylococcus aurous*

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ABSTRACT: Antibiotic resistance in pathogens has become a serious problem world wide. Therefore, the search for new antibiotics for drug resistant pathogens is an important endeavour. The present study was undertaken with the objective of using Nano particle to study the methods to overcome these problems. Poly lactic-co-glycolic acid (PLGA) Nano particle containing methicillin were prepared by the A double emulsion process diffusion method. The resulting nanoparticles were analyzed for their, particle size and size distribution, drug loading and entrapment efficiency, thermal properties with XRD apparatus and antibacterial activity by disc diffusion and MIC antibiogram methods. The nanoparticles prepared in this study were spherical, with an average particle size of 87-136nm. Drug release studies performed in phosphate buffer at pH 7.4 indicated slow release of meticillin from 1day to 4 days. On antibacterial analysis, the minimum inhibitory concentration nanoparticles was at least two times lower than that of the free drug but in early days the results was in contrast . According to the size and surface properties of the prepared particles, it may be concluded that they are a good formulation for escape from different mechanisms of antibiotic resistance in MRSA infections.

Keywords: Drug delivery, PLGA, MRSA, MIC, antibiotic resistance

INTRODUCTION

Staphylococcus aureus, versatile human pathogens responsible for nosocomial and community-associated infections is associated with high morbidity and mortality rate. However, emerging reports revealed that increase rate of hospital-acquired infections, are mostly due to antibiotic-resistant pathogens. Of the resistant pathogens that had attracted public health interest worldwide is methicillin-resistant *S. aureus* (MRSA). It is major cause of nosocomial infection and colonization, resulting in morbidity and mortality. Consequential effect of MRSA infection had resulted in, prolonged hospitalization, increased in medical expenses, and difficulty in patient treatment and management. In US hospitals, MRSA accounts for mostof invasive *S. aureus* infections, with high fatality rate (Okon *et al.,* 2013).

The resistance to antimicrobial agents is an increasingly global problem worldwide, especially among nosocomial pathogens. Staphylococci have become one of the most common causes of noso comial infections. Multidrug-resistant staphylococci pose a growing problem for human health. The rise of drug-resistant virulent strains of *Staphylococcus aureus*, particularly methicillin-resistant *S. aureus* (MRSA) is a serious problem in the treatment and control of staphylococcal infections (Harrison and Holcomb 2008). An indiscriminate use of antimicrobial agents often leads to emergence of resistant microorganisms to one or several of them. Since the pattern of sensitivity is constantly changing, monitoring of the antimicrobial susceptibilities becomes more important. It provides information on the pathogenic organisms isolated from patients as well assist in choosing the most appropriate antimicrobial therapy till the culture reports become available (Deshpande *et al.,* 2011).

The unique characteristic of MRSA strains is the multidrug resistance pattern to β-lactam and other classes, due to acquisition of mecA gene, key genetic determinant located on the staphylococcal cassette chromosome (SCCmec) (Hiramatsu *et al.,* 2001).

The late Victorian period witnessed a number of observations on microbial antagonism which was the ability of one microorganism to kill or limit the growth of another. But the most famous observation came in 1928 when Alexander Fleming discovered Penicillin, an antibiotic produced by Penicillium mould against *Staphylococcus aureus*.

But the irrational and indiscriminate use of antibiotics in agriculture and to treat common infections ultimately led to the problem of antibiotic resistance. For example rampant use of methicillin has led to the development of Methicillin Resistant *Staphylococcus aureus* (MRSA) which is still a major concern in hospitals whereas indiscriminate use of third generation antibiotics such as Vancomycin and Cephalosporin has led to new strains of Vancomycin resistant Enterococcus. It has been observed that corrective measures such as optimising the use of antibiotics might not decrease instances of antibiotic resistance in the near future. Therefore these drawbacks led scientists to focus on developing antimicrobial agents to which microorganisms might not develop resistance. Thus came nanoparticles into limelight (Guillemot, 1999, Dahms *et al.,* 1998).

The predominant role of polymeric nanoparticles in drug delivery system is to carry the drug molecules, to protect drugs from degradation, and to control drug release. Therapeutically used polymeric nanoparticles are composed of biodegradable or biocompatible materials, such as poly (ε-caprolactone) (PCL), poly (lactic acid) (PLA), poly (lactic-co-glycolic acid) (PLGA), alginic acid, gelatin and chitosan (Abhishek *et al.,* 2013).

Nanoencapsulation of therapeutic agents increases their efficacy, specificity and targeting ability. Nanocarriers (NCs) protect their payload from premature degradation in the biological environment, enhance bioavailability, and prolong presence in blood and cellular uptake (Avnesh *et al.,* 2014).

Polymeric drug delivery devices have numerous advantages compared to conventional dosage forms, such as improved therapeutic effect, reduced toxicity and convenience. However, the conventional polymeric drug delivery systems like nano- or microspheres, liposomes and hydrogels often exhibit the problem of a high burst release at the beginning. Electrospinning is a versatile technique for the generation of drug loaded polymeric nanofiber nonwovens for the controlled release of the incorporated drugs (Goldberg and Langer 2007).

PLGA nanoparticles (NPs) protect the therapeutic agents and increase their stability and can be used for controlled delivery of therapeutics with improved pharmacokinetic and pharmacodynamic profile. Furthermore, PLGA is approved by United States Food and Drug Administration (FDA) and European Medicine Agency (EMA) for various drug delivery formulations. However, PLGA NPs, suffer from a significant limitation due to their high level of opsonization by reticuloendothelial system (RES) (Manoochehri *et al.,* 2013).

The release profile of PLGA nano particles can be divided into 4 different phases: initial burst, induction period, slow release period and final release period Polymers containing 50:50 ratios of lactic and glycolic acid have faster hydrolytic activities than those with other ratios of the monomers. PLGA nanoparticles can be used safely for oral, nasal, pulmonary, parenteral, transdermal and intra-ocular routes of administration. The PLGA nanoparticles can be prepared by different techniques (Abdullahi *et al.,* 2013).

In the present work, we conduct encapsulation of antibiotic drug and theoretical studies to investigate how PLGA encapsulated antibiotic escape from snare of antibiotic resistance Mechanisms. The objective of this paper is to report the conjugation of methicillin - PLGA and study the antimicrobial effects of encapsulated drug.

MATERIAL AND METHODS

This study was conducted to examine the eduction of *Staphylococcus aureus* resistant to methicillin is made of nano-medicine. A) Strains of bacteria: methicillin-resistant strains of MRSA from the Pasteur Institute of Iran were purchased. (ATCC25923) then the 0.5 CC of BHI liquid is injected into the vial and then at 37°C for half an hour to put the contents of the syringe is pulled and cultured on blood agar and monitol salt agar. B)

A. Synthesis and characterization of PLGA-methicillin nanoparticles

Nanoparticles were prepared by emulsion solvent evaporation technique as described by Xua et al (2005), with different ratios of PLGA 50:50 1 gr nanoparticles PLGA were purchased from Sigma Corporation of PLA to PGA 50-50%. Also 1gr methicillin was purchased from.....co. Initially, 50 mg PLGA solution in 2 ml of dichloromethane. Then meticilin added to nanoparticle solution and dichloromethane on a rotating magnetic.

This solution name is the primary emulsion of water / oil. This emulsion in three stages, each lasting 10 seconds of sonication. Then added to a solution of 2ml PVA 5% and three times was Sonic. After sonication, to the secondary emulsions 4 mL of polyvinyl alcohol 0/25% added and for three hours on a rotating magnetic field and was next to the ice until dichloromethane evaporate. Nano particles are centrifuged for 40 minutes at a speed of 16000rpm. The organic solvent was allowed to evaporate during 4 hours at room temperature under gentle mixing. Finally, the nanoparticles were collected by centrifugation and freeze-dried after three times washing with deionized water (the precipitate was washed three times, each time with 10 ml of water was given. in the final stage of nanoparticles was lyophilized). The effects of encapsulation process on the chemical group and the interaction between the components was studied by performing by XRD technique using GNR Corporation Italy, model MPD300.

Agar Dilution. Agar dilution is the reference method that has been established by the CLSI for susceptibility testing of anaerobic bacteria. This procedure involves making a series of agar plates, each containing a specific concentration of an antimicrobial agent.

This procedure is usually used for periodic testing to monitor resistance in anaerobic bacteria at a facility or for testing a new antimicrobial agent to determine its activity against a wide range of species. This is the gold standard to which all other methods are compared. If any other method can prove its equivalency to this method, it is acceptable for use in the clinical lab. It is not the function of the CLSI to validate every susceptibility method available, so there will always be other methods.

Antimicrobial susceptibility pattern was studied by Modified Kirby-Bauer disc diffusion method with a panel of 2 drugs as per the Clinical Laboratory Standard Institute (CLSI) guidelines and sensitivity pattern was noted.

The diameter of zone of inhibition of growth was recorded and interpreted by the criteria of CLSI. *E. coli*, *Klebsiella* and *Proteus* spp.

S. aureus ATCC 25923 (mecA negative) and ATCC 43300 (mecA positive) were used for the quality control of all the tests. Mueller-Hinton agar (MHA) plates were overlaid with the saline suspension of a strain (turbidity = 0.5 McFarland standard) and antibiotic discs of methicillin and PLGA-methicillin (5µg) After 24 and 48 hours of incubation at 35ºC, all plates were read according to standard procedure .

B. Validation of McFarland standard turbidity

Absorbency method: Read the optical density of the standard on a spectrophotometer with a 1^{-cm} light path using a matched set of cuvettes. The absorbency should be read at 625nm. 0.5 McFarland standard should have a reading of 0.08 to 0.10, 1 McFarland standard should have a reading of 0.16 to 0.20available that CLSI does not describe or discuss.

Microbroth Dilution. Of the procedures described by the CLSI, this is the most practical one for the clinical microbiology laboratory to use when testing a small number of isolates against a given set of antibiotics.

Minimum inhibitory systems. MACRO MIC tests: Samples (1 ml) of Mueller-Hinton broth containing serial dilutions of an antimicrobial agent
(methicillin, PLGA-methicillin) were used. The rack of inoculated tubes was gently shaken to mix the contents, and tubes were incubated in a 35°C air incubator for 22 to 24 h. Tubes were then individually shaken by hand to resuspend the contents, and a 0.01-ml calibrated loopful was streaked over a quadrant of a 90-mm blood agar plate (tryptic soy agar base with 5% sheep blood). Colonies on subculture plates were counted after 24 h at 35°C.

Preparation of antibiotic Stock solution. Antibiotic stock solution can be prepared by commercially available antimicrobial powders (with given potency) and the amount needed and the diluents in which it can be dissolved can be calculated by using either of the following formulas to determine the amount of powder (1) or diluent (2) needed for a standard solution:

$$1.\ weight = \frac{volume(ml) + concentration(\frac{\mu g}{ml})}{potency(\frac{\mu g}{mg})}$$

Or

$$2.\ volume = \frac{weight(mg) + potency(\frac{\mu g}{mg})}{concentration(\frac{\mu g}{ml})}$$

Prepare antimicrobial agent stock solutions at concentrations of at least 1000 µg/mL (example: 1280 µg/mL) or 10 times the highest concentration to be tested, whichever is greater. Because microbial contamination is extremely rare, solutions that have been prepared aseptically but not filter sterilized are generally acceptable. If desired, however, solutions may be sterilized by membrane filtration. Dispense small volumes of the sterile stock solutions into sterile glass, polypropylene, polystyrene, or polyethylene vials; carefully seal; and store (preferably at -60 °C or below, but never at a temperature warmer than -20 °C and never in a self-defrosting freezer). Vials may be thawed as needed and used the same day.

Preparation of antibiotic dilution range. Use sterile 13- x 100-mm test tubes to conduct the test. If the tubes are to be saved for later use, be sure they can be frozen. Close the tubes with loose screw-caps, plastic or metal closure caps, or cotton plugs. Prepare the final twofold (or other) dilutions of antimicrobial agent volumetrically in the broth. A minimum final volume of 1 ml of each dilution is needed for the test.

Preparation of Inoculum. Prepare the inoculum by making a direct broth suspension of isolated colonies selected from an 18- to 24-hour agar plate (use a nonselective medium, such as blood agar).Adjust the suspension to achieve a turbidity equivalent to a 0.5 McFarland turbidity standard. This results in a suspension containing approximately 1 to 2 × 108 colony forming units (CFU)/mL. Compare the inoculum tube and the 0.5 McFarland standards against a card with a white background and contrasting black lines. Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in broth so, after inoculation, each tube contains approximately 5 × 105 CFU/mL. This can be accomplished by diluting the 0.5 McFarland suspensions 1:150, resulting in a tube containing approximately 1 × 106 CFU/mL. The subsequent 1:2 dilution in step 3 brings the final inoculum to 5 × 105 CFU/mL.

Theoretical study of penicillin binding protein in *staphylococcus aureus* methicillin resistance: creating structure -based pharmacophore models:

For automated generation of pharmacophore models favored PDB complex was imported to ligand Scout software.

A pharmacophore is defined as an ensemble of universal chemical feature that characterize a specific mode of action of a ligand in the active site of the macromolecule in 3D space. In this research was used penicillin binding protein: PDB (1MWT) with two ligand. The program automaticaly searches for interaction between ligand and macromolecule.

RESULTS AND DISCUSSION

A. Structural characterization of PLGA-methicillin nanoparticles

The structural analysis of the powders by means of X-ray diffraction (XRD), the results of which are displayed in Fig. 1-3, demonstrates that most methicillin particles were covered with PLGA during the encapsulation process, as evident from the effect of increased amorphousness that PLGA has on the XRD pattern of pure methicillin. There is a growing concern about the rapid rise in resistance of S. *aureus* to antimicrobial agents. Over the last few decades, the applications of nanotechnology in medicine have been extensively explored in many medical areas, especially in drug delivery.

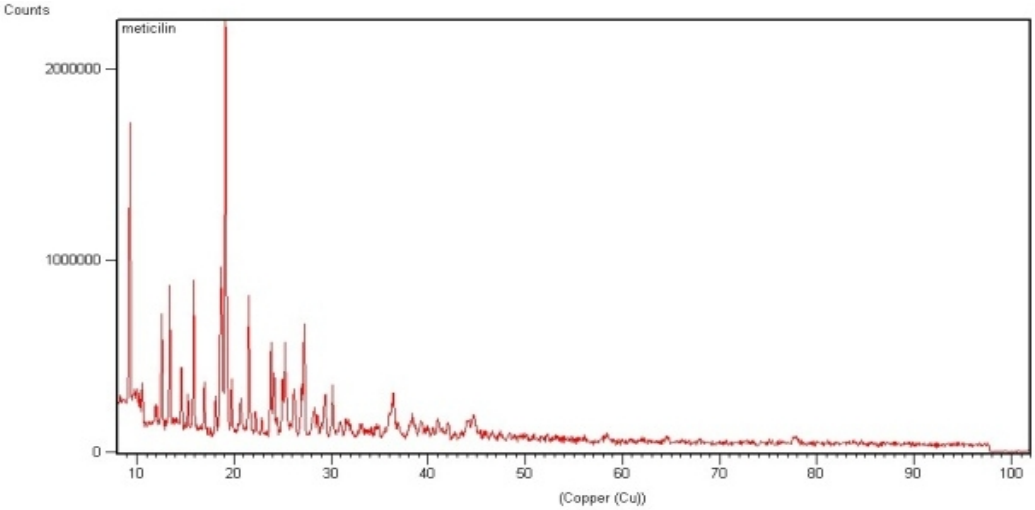

Fig. 1. XRD spectrum of methicillin.

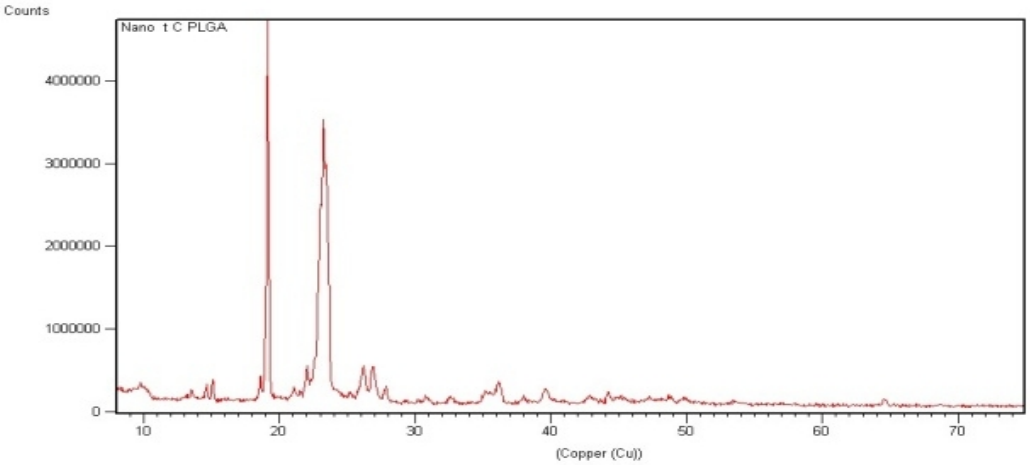

Fig. 2. XRD spectrum of PLGA.

Nanotechnology concerns the understanding and control of matters in the 1-100 nm range, at which scale materials have unique physicochemical properties including ultra small size, large surface to mass ratio, high reactivity and unique interactions with biological systems (Zhang *et al.*, 2008).

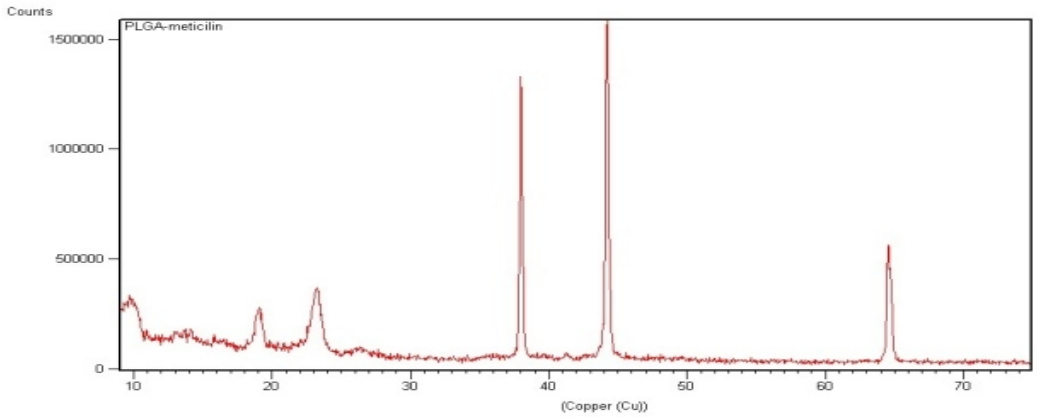

Fig. 3. Spectra of PLGA-methicillin.

By loading drugs into nanoparticles through physical encapsulation, adsorption, or chemical conjugation, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts. Many advantages of nanoparticle-based drug delivery have been recognized, including improving serum solubility of the drugs, prolonging the systemic circulation lifetime, releasing drugs at a sustained and controlled manner, preferentially delivering drugs to the tissues and cells of interest, and concurrently delivering multiple therapeutic agents to the same cells for combination therapy (Davis *et al.*, 2008).

Poly (lactic-co-glycolic acid) (PLGA) is one of the most successfully developed biodegradable polymers. Among the different polymers developed to formulate polymeric nanoparticles, PLGA has attracted considerable attention due to its attractive properties: (i) biodegradability and biocompatibility, (ii) FDA and European Medicine Agency approval in drug delivery systems for parenteral administration, (iii) well described formulations and methods of production adapted to various types of drugs e.g. hydrophilic or hydrophobic small molecules or macromolecules, (iv) protection of drug from degradation, (v) possibility of sustained release, (vi) possibility to modify surface properties to provide stealthness and/or better interaction with biological materials and (vii) possibility to target nanoparticles to specific organs or cells (Danhier *et al.*, 2012).

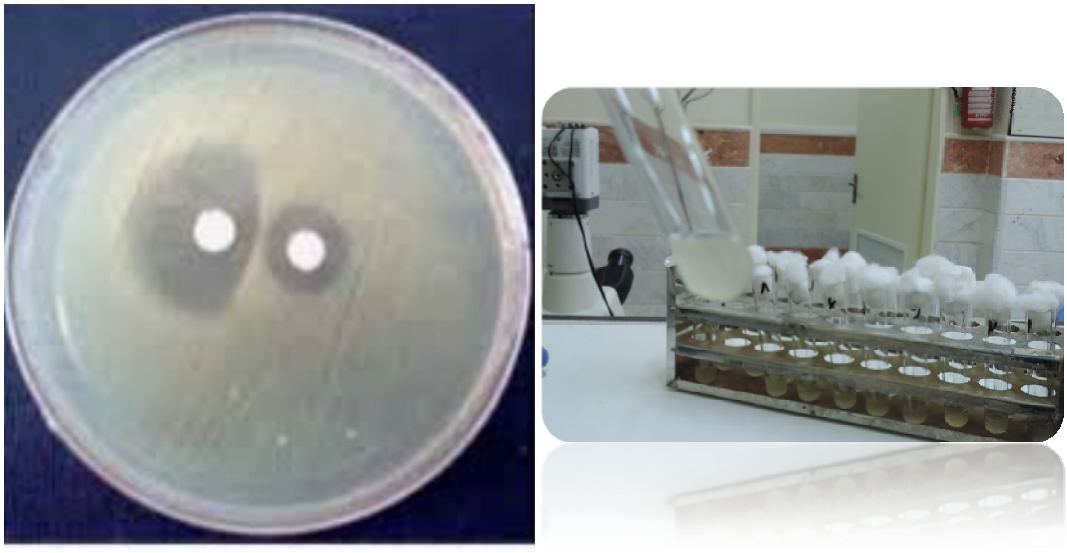

Fig. 4. Disc diffusion tube MIC antibiogram of methicillin and nanoparticle PLGA-methicillin.

The MIC of PLGA formulations and methicillin powder on *S. aureus* was reported in Fig. 4. Drug free polymeric nanoparticles showed less antibacterial activity which indicates that nanosphere is protective effect in front of the antibiotic devasting enzymes. The MIC of methicillin loaded PLGA NPs were 2 times less on *S. aureus* compared to methicillin solution at a concentration of time. Consequently, in vitro anti-microbial activity of methicillin PLGA NPs is better than the commercialized drug formulations. PLGA NPs may have been resulted from higher bacterial adhesion of the NPs. In other studies enhanced antimicrobial activity from PLGA NPs containing antimicrobial agents has been reported (Peng *et al.*, 2008, Grislain *et al.*, 1983). The improved the therapeutic effect of the nanoparticulate antibacterial delivery systems may the result of increased drug concentration nearby its target. Moreover, improved penetration of NPs from biological membranes is also generally accepted. For methicillin to be effective it should pass the bacterial membrane to reach its intra cellular site of action.

Recently, nontraditional antibiotic agents have been of tremendous interest in overcoming resistance that is developed by several pathogenic microorganisms against most of the commonly used antibiotics. Especially, several classes of antimicrobial nanoparticles (NPs) and nano sized carriers for antibiotics delivery have proven their effectiveness for treating infectious diseases, including antibiotics resistant ones, in vitro as well as in animal models (Huh and Kwon 2011).

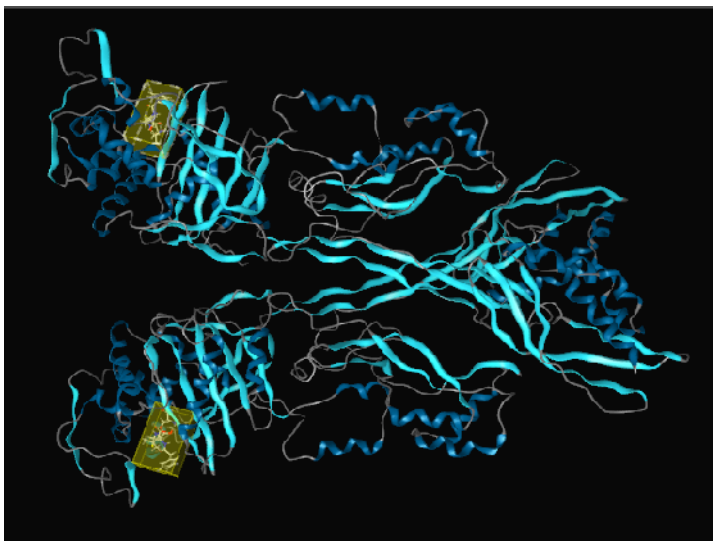

Fig. 5. Structure of penicillin G Acyl-penicillin binding protein 2A from methicillin resistant *Staphylococcus aureus* strain.

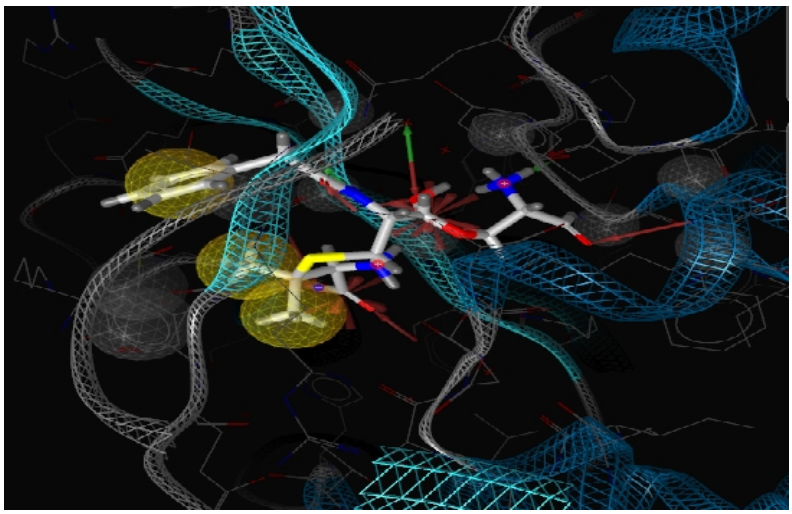

Fig. 6. Selection of ligand/active site of Acyl-penicillin binding protein.

Typically, antimicrobials kill bacteria by binding to some vital compounds of bacterial metabolism, thereby inhibiting the synthesis of functional biomolecules or impeding normal cellular activities. For instance, β-lactams such as penicillins and cephalosporins inhibit bacteria cell wall synthesis (Walker, 2000).

Despite the great progress in antimicrobial development, many infectious diseases, especially intracellular infections, remain difficult to treat. One major reason is that many antimicrobials are difficult to transport through cell membranes and have low activity inside the cells, thereby imposing negligible inhibitory or bactericidal effects on the intracellular bacteria. In addition, antimicrobial toxicity to healthy tissues poses a significant limitation to their use (Zhang *et al.*, 2010).

Methicillin resistant *S. aureus* has become an enormous problem for health care providers because it is hard to treat and is sometimes called super bug. Multiple studies have been carried out on growing concern over multidrug resistance including India. MRSA is becoming a problem in paediatric population including hospital setting.

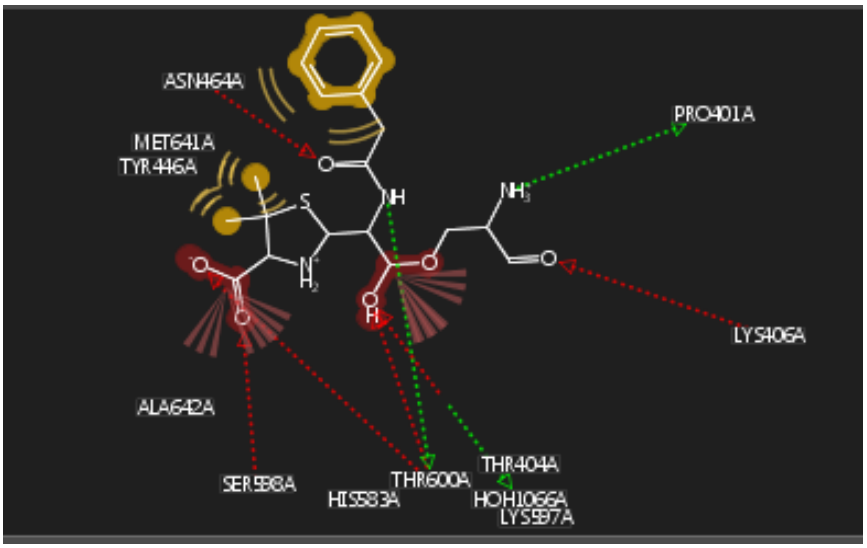

Fig. 7. Penicillin G -Acyl serine.

Table 1: Chemical feuture of penicillin G -Acyl serine (ligand).

Formula & mol. weight	**Size & flexibillity**				**Polarity & chemical features**					
C19H28N3O16S1 &42512	atom	rings	Rotatable bonds	Aromatic atoms	Clogp	TpsA	Acceptanc	donor	Neg ionizable	Pos ionizable
	57	2	14	6	-5.251	237.3	7	4	2	2

The previous inclination of MRSA is in high intensity in the surgical and intensive care services, where antibiotic usage is the greatest. According to our study, there is high occurrence of MRSA in surgical wound infection, due to overcrowding, workload, and understaffing of wards. The MRSA could be prevented by identifying and screening MRSA carriers inside high-risk wards. Findings of the current study support, research for finding of effective antibiotics it is necessary.

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